Smooth muscle cell migration and proliferation are mediated by distinct phases of activation of the intracellular messenger mitogen-activated protein kinase

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Purpose: **Mitogen-activated protein kinase (MAPK) is a ubiquitous signaling protein that has been associated with cellular proliferation; however, its role in cellular migration has not been established. In this study, we investigate the role of MAPK in platelet-derived growth factor (PDGF)–induced migration and proliferation of human vascular smooth muscle cells (SMCs).**
Methods: **SMC migration was measured using a microchemotaxis assay (4 hours), and proliferation was assessed using ^{3}H-thymidine uptake and cell counts. PD098059 was used as a specific noncompetitive inhibitor of MAPK activation.**
Results: **Coincubation of SMCs with PD098059 resulted in significant inhibition of PDGF-BB (5 ng/ml)–induced SMC chemotaxis and proliferation. The IC_{50} for both processes was approximately 10 μmol/L with complete inhibition at 50 μmol/L. Stimulation of SMCs with PDGF produced an early peak in MAPK activity followed by a plateau of activity that persisted for 24 hours. We hypothesized that variations in the temporal activation of MAPK might explain the action of this enzyme on these two disparate cellular events. By adding PD098059 at intervals after stimulation of SMCs with PDGF, we demonstrated an association between MAPK activity within the first 15 minutes and SMC migration, whereas MAPK activity between 1 and 4 hours was associated with SMC proliferation.**
Conclusions: **MAPK activity is essential for both SMC migration and proliferation, and distinct phases of enzyme activation are required to stimulate these two discrete cellular events. Inhibition of this signaling protein may prove to be a useful method for preventing intimal hyperplasia. (J Vasc Surg 1998;27:117-25.)**

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The success of vascular reconstruction remains limited by restenosis that develops as a result of intimal hyperplasia. Vascular smooth muscle cell (SMC) migration and proliferation are both necessary events that contribute to the formation of intimal plaque.[1] SMCs at sites of arterial injury are exposed to and influenced by a number of growth factors, extracellular matrix proteins, and cytokines that mediate these pathologic processes.[2] In some instances, the same factor can stimulate both migration and proliferation, the predominant example being the potent agonist platelet-derived growth factor (PDGF).

Strategies to inhibit the formation of intimal hyperplasia include the use of agents that alter either

the extracellular or intracellular events responsible for the pathologic behavior of SMCs. Success with the latter approach requires an understanding of the intracellular signaling pathways that mediate SMC migration and proliferation. The ultimate cellular events that are required for these two processes are distinct; migration requires cytoskeletal mobilization, and proliferation is dependent on nuclear activation. However, in the case of PDGF, there must be an initial common signaling pathway that at some level diverges into pathways that are specific for each process. Second messenger proteins that are specific for migration or proliferation have been identified; however, the precise point of divergence is not known.

Mitogen-activated protein kinase (MAPK) is a ubiquitous intracellular serine-threonine kinase that plays a fundamental role in many cellular processes. A variety of MAPK isotypes have been isolated; however, the most widely studied are the 44 and 42 kd proteins designated as extracellular signal-regulated protein kinases (ERK), ERK-1 and ERK-2, respectively. MAPK activity is stimulated by a number of growth factors, differentiating agents, cytokines, and vasoactive substances, and a wide range of substrates for MAPK have been identified.[3]

We and others have demonstrated that multiple growth factors, including PDGF-BB, activate MAPK in human vascular SMCs.[4] Using an assay of MAPK activity, we found that PDGF-BB activated MAPK for an interval up to 24 hours, with an initial peak in activity at 10 minutes, followed by a plateau of approximately 45% of this peak that persisted for the remainder of the 24-hour period. We have also demonstrated an association between MAPK activation and SMC proliferation.[4,5] For both basic fibroblast growth factor (bFGF) and PDGF, there was a precise correlation between the ability of these agonists to activate MAPK and their potential to induce a proliferative response. However, a direct cause/effect relationship between MAPK activation and SMC proliferation has not been definitively established.

The role of MAPK in cellular migration is less well understood. Bornfeldt et al.[6] hypothesized that MAPK is not necessary for SMC migration. They found PDGF to be both a potent stimulus of MAPK activity and a strong agonist of SMC chemotaxis. However, insulin-like growth factor I, which was also a potent agonist of SMC migration, stimulated very little MAPK activity. Recent reports have addressed the importance of MAPK in migration of other cell types. Klemke et al.[7] found that MAPK is necessary for migration of a carcinoma cell line, whereas Sa and coworkers[8] have implicated MAPK signaling in endothelial cell motility. In contrast, Knall et al.[9] revealed that MAPK is not necessary for migration of neutrophils.

PD098059 is a selective, noncompetitive, reversible inhibitor of the MAPK signaling pathway characterized by Alessi and collaborators.[10] This inhibitor, which is cell-permeable, blocks the activation of MAPK by MAPK kinase (MAPKK) and has been used to define the role of MAPK in intracellular signaling responses to nerve growth factor, insulin, and growth hormone. In concentrations less than 100 μmol/L, PD098059 does not appear to effect other intracellular pathways, including raf, protein kinase C, phospholipase C, phosphatidylinositol 3-kinase, cyclic adenosine monophosphate–dependent kinase, v-Src, p70 S6 kinase, and the epidermal growth factor (EGF) and PDGF receptor kinases.[11-14] Moreover, PD098059 is also selective for ERK-1 and ERK-2 and has no effect on other MAPK isotypes, including p38/reactivating kinase (p38/RK) and p54 stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK).[15]

Using this inhibitor, we explored the role of MAPK in migration and proliferation of human vascular SMCs. We found that MAPK is required for both processes. Moreover, our data suggest that distinct phases of MAPK activation are required to stimulate these two discrete cellular events.

METHODS

Materials

Human recombinant PDGF-BB was obtained from Upstate Biotechnologies, Inc. (Lake Placid, N.Y.). The MAPKK inhibitor PD098059 (2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one) was obtained from Research Biochemicals International (Natick, Mass.). Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered saline solution (PBS), fetal bovine serum, trypsin–ethylenediamine tetraacetic acid (EDTA), penicillin/streptomycin/Fungizone solution, and N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) were obtained from Gibco BRL Life Technologies (Gaithersburg, Md.). L-glutamine was obtained from BioWhitaker (Walkersville, Md.). [Methyl-^{3}H] thymidine and γ-^{32}P-ATP (6000 Ci/mmol) were obtained from DuPont New England Nuclear (Boston, Mass.). Rabbit antirat ERK-2 MAPK antibody, which has substantial cross-reactivity to human and other species, was obtained from Santa Cruz Biotechnology (Santa Cruz,

Calif.). Polycarbonate 8 μm-pore membranes were obtained from Poretics Corp. (Livermore, Calif.). Rabbit brain myelin basic protein, adenosine 5'-triphosphate (ATP, disodium salt), the smooth muscle–specific actin immunostaining kit, and other stock chemicals were purchased from Sigma Chemical Company (St. Louis, Mo.). Protein A-sepharose was obtained from Pharmacia Biotech (Uppsala, Sweden).

Cell culture

Human SMCs were explanted from saphenous vein harvested at the time of aortocoronary or peripheral arterial bypass grafting, as previously described.[16] Endothelial and adventitial layers were gently removed from segments of vein, and fragments of the medial layer were placed onto tissue culture plates. Outward-growing SMCs were harvested and subcultured in DMEM supplemented with 10% fetal bovine serum, 25 mmol/L HEPES, 40 U/ml penicillin G, 40 μg/ml streptomycin, 100 ng/ml amphotericin B, and 4.8 mmol/L L-glutamine at 37° C and 5% CO_2 in room air. Cells in passages 1 through 5 were used for all experimentation. SMC identity was verified by immunostaining with antihuman α-actin antibody and by a characteristic hill-and-valley growth pattern.

Migration assay

SMCs were grown to confluence and then made quiescent in serum-free DMEM for 72 hours. Cells were washed in PBS, harvested using 0.05% trypsin-EDTA, and resuspended in serum-free DMEM. Migration was assayed for 4 hours at 37° C in a 48-well microchemotaxis chamber (Neuroprobe, Cabin John, Md.) with upper and lower wells separated by a polycarbonate 8 μm-pore membrane. For all assays, SMCs in the presence or absence of PD098059 were seeded at a density of 50,000 per well (6000/mm^2) into the upper well of the chamber. PDGF-BB diluted in DMEM was added to the lower well. Vehicle in DMEM served as a control.

After completing the assay, the membrane was washed in PBS, fixed in 70% ethanol for 20 minutes at –20° C, and stained in hematoxylin overnight. The upper side of the membrane was carefully scraped using a cotton swab, leaving the cells that had migrated to the lower surface undisturbed. The membrane was then mounted onto a microscope slide, and migration in each well was assessed with light microscopy by counting the number of cells in five independent, random, high-power fields at 200× magnification.

Attachment assay

Attachment assays were performed for 2 hours at 37° C in a 48-well microchemotaxis chamber. For all assays, SMCs in serum-free DMEM were seeded at a density of 2000 cells per well (240/mm^2) into the upper well of the chamber. Cells in the presence or absence of the inhibitor PD098059 were added to the upper well of the chamber, and after a 2-hour period the membranes were washed in PBS, fixed in ethanol, and stained in hematoxylin as described for the migration assay. Attachment to both the upper and lower surfaces of the membrane was assessed with light microscopy at 200× magnification by counting the total number of cells on both sides of the membrane.

Proliferation assay

DNA synthesis. SMCs were seeded into a 24-well plate at a concentration of 10^4 cells/well and were allowed to attach overnight. These cells were starved in serum-free DMEM for 72 hours and then stimulated for 24 hours with PDGF-BB (5 ng/ml) in the presence or absence of PD098059. During the final 4 hours of the assay, 2 μCi of [methyl-^{3}H]-thymidine was added to each well. Cells were then rinsed with PBS and treated with 10% (w/w) trichloroacetic acid. After washing with 95% ethanol, the trichloroacetic acid–insoluble fraction was solubilized with 0.5 N NaOH, and radioactivity was measured using a scintillation counter.

Cell counts. SMCs were seeded at a concentration of 3×10^4 cells/well in six-well plates and were allowed to attach overnight. Cells were then starved in serum-free DMEM for 72 hours and were incubated for an additional 72 hours in DMEM containing PDGF-BB (5 ng/ml) in the presence or absence of PD098059. At the end of the 72-hour incubation, cells were removed with trypsin-EDTA and quantified with a Coulter Counter (Coulter Electronics, Hialeah, Fla.).

MAPK immunoprecipitation/activity assay

SMCs were allowed to grow to confluence in 75 cm^2 plates and were rendered quiescent in serum-free DMEM for 72 hours. After stimulation with PDGF, in the presence or absence of PD098059, SMCs were lysed with radioimmunoprecipitation assay (RIPA) buffer (10 mmol/L Tris-HCl, pH 7.5, 1.0% Triton X-100, 0.5% NP 40, 150 mmol/L NaCl, 50 mmol/L NaF, 0.5 mmol/L Na orthovanadate, 1.0 mmol/L EGTA, 1.0 mmol/L EDTA, 0.5 mmol/L phenylmethylsulfonyl fluoride) for 20 minutes on ice. Cell lysates were collected into

microcentrifuge tubes, vortexed, and centrifuged at 4° C for 20 minutes. Protein concentration was measured in the supernatant and equalized for all samples. Cell lysates were then coincubated with rabbit antirat ERK-2 MAPK antibody at a concentration of 2 μg/ml for 2 hours. The antibody-MAPK complex was then conjugated to protein A-sepharose overnight, centrifuged, and washed three times in RIPA buffer and twice in RIPA containing 500 mmol/L NaCl. The immunoprecipitated pellet was resuspended in an equal volume of kinase buffer containing 10 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 10 mmol/L $MgCl_2$, for a final volume of 50 μl. Five μCi of γ-^{32}P-ATP, 8 μg myelin basic protein, and 10 μmol/L ATP were added to each sample. The reaction was allowed to continue for 30 minutes at 30° C and was then halted by the addition of 4x Laemmli buffer (250 mmol/L Tris-HCl, pH 6.8, 8% sodium dodecylsulfate [SDS], 20% glycerol, 10% 2-mercaptoethanol). Samples were then boiled for 5 minutes, quenched on ice for an additional 5 minutes, and then subjected to sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Dried gels were analyzed by autoradiography.

Isolation of temporal phases of MAPK activation

To temporally isolate MAPK activity, PD098059 (50 μmol/L) was added coincident with and at intervals after PDGF stimulation. For example, the addition of this inhibitor at a point 15 minutes after stimulation of SMCs with PDGF allowed the occurrence of the first 15 minutes of MAPK activation (i.e., the early peak) but eliminated all subsequent activity (i.e., the plateau phase). Thus the migratory or proliferative response of SMCs subjected to these circumstances was related only to the MAPK activity that occurred during this initial 15-minute period. Longer and shorter periods of MAPK activation were isolated by adding the inhibitor at earlier or later time intervals.

Statistical analysis

Statistical comparisons were made using an unpaired Student's *t* test with Statview (BrainPower Inc., Calabasas, Calif.) software on a Macintosh System (Apple Computer, Inc., Cupertino, Calif.). For all comparisons, a p value less than 0.05 was considered significant. The reported results are a representative example from one cell line; however, we confirmed these results using cells from at least three different donors.

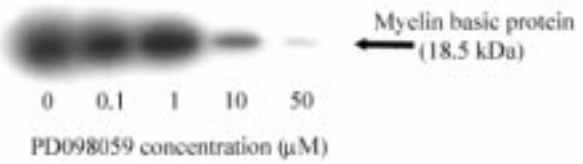

Fig. 1. Effect of PD098059 on PDGF-BB–induced activation of MAPK. Quiescent SMCs were simultaneously incubated with varying concentrations of PD098059 and PDGF-BB (5 ng/ml) for 10 minutes. MAPK activity, as measured by phosphorylation of its substrate myelin basic protein, was determined using immunoprecipitation and SDS-PAGE techniques as described in Methods. This experiment was repeated three times using cells derived from different saphenous veins. A representative example is displayed.

RESULTS

MAPK activation by PDGF-BB in SMCs is inhibited by PD098059. To evaluate whether the inhibitor, PD098059, could block MAPK activity in intact human vascular SMCs, we measured MAPK in quiescent SMCs 10 minutes subsequent to their simultaneous stimulation with PDGF-BB (5 ng/ml) and increasing concentrations of PD098059. PD098059, in concentrations ranging from 0.1 to 1.0 μmol/L, had no effect on MAPK activity, whereas 10 μmol/L partially eliminated and 50 μmol/L completely eliminated the increase in MAPK activity produced by PDGF (Fig. 1). Thus PD098059 was an immediate and effective inhibitor of PDGF-induced MAPK activation. We then evaluated whether PD098059 (50 μmol/L), when added to SMCs in a delayed fashion after PDGF stimulation, eliminated all subsequent MAPK activity. PD098059 was added at intervals after stimulation of SMCs with PDGF, and MAPK activity was measured 5 minutes subsequent to the addition of the inhibitor. When added at time points ranging from 5 minutes to 4 hours after stimulation of SMCs with PDGF, PD098059 consistently eliminated all subsequent MAPK activity (data not shown).

MAPK activation is necessary for SMC chemotaxis. To determine whether MAPK activation is necessary for SMC migration, chemotaxis to PDGF was measured in the presence of increasing concentrations of PD098059. PDGF-BB alone (5 ng/ml) consistently produced a 3.5- to 4.5-fold increase in SMC chemotaxis as measured using a 4-hour microchemotaxis chamber assay. The concentrations of PD098059 required to inhibit chemotaxis were similar to those required to inhibit MAPK

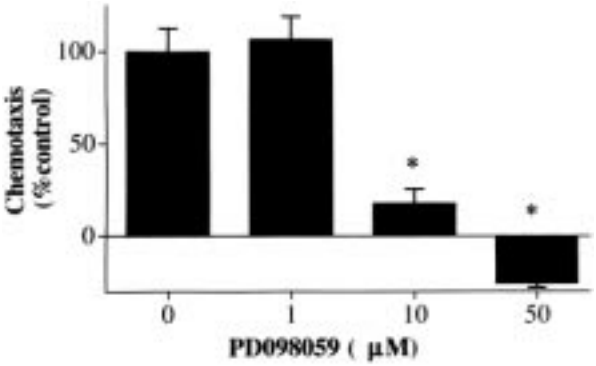

Fig. 2. Effect of PD098059 SMC chemotaxis. Quiescent SMCs were stimulated with PDGF (5 ng/ml) in the presence of increasing concentrations of PD098059. Chemotaxis was determined using a 4-hour microchemotaxis chamber assay as described in Methods. Results are expressed as mean percent of control (PDGF-stimulated) chemotaxis ± SD. All experiments were repeated three times using cells derived from different saphenous veins. A representative example is displayed.

activity. Low concentrations of PD098059 (0.1 to 1 μmol/L) had no effect on chemotaxis, whereas 10 μmol/L partially inhibited (~80%) and 50 μmol/L completely inhibited the chemotactic effect of PDGF (Fig. 2). The IC_{50} for the inhibitory effect of PD098059 on PDGF-induced SMC chemotaxis was 8 μmol/L. In separate assays, PD098059 had no effect on SMC attachment (data not shown). Thus the diminished chemotaxis that we observed in these assays was not the result of an effect of PD098059 on attachment of SMCs to the polycarbonate membranes of the microchemotaxis chambers.

MAPK activation is necessary for SMC proliferation. Our previous studies have suggested a positive association between MAPK activation and SMC proliferation.[4,5] To verify a causal relationship between these two events, DNA synthesis (^{3}H-thymidine incorporation) was measured in SMCs stimulated with PDGF-BB (5 ng/ml) coincubated with increasing concentrations of PD098059. PDGF produced a sixfold increase in DNA synthesis that was inhibited by PD098059 in a concentration-dependent fashion (Fig. 3, *A*). The IC_{50} for the effect was 10 μmol/L, and there was complete inhibition of proliferation with a PD098059 concentration of 50 μmol/L. PD098059, in similar concentrations, inhibited the stimulatory effect of PDGF on SMC proliferation as measured by cell counts (Fig. 3, *B*). Thus activation of MAPK is clearly necessary for PDGF-induced SMC proliferation.

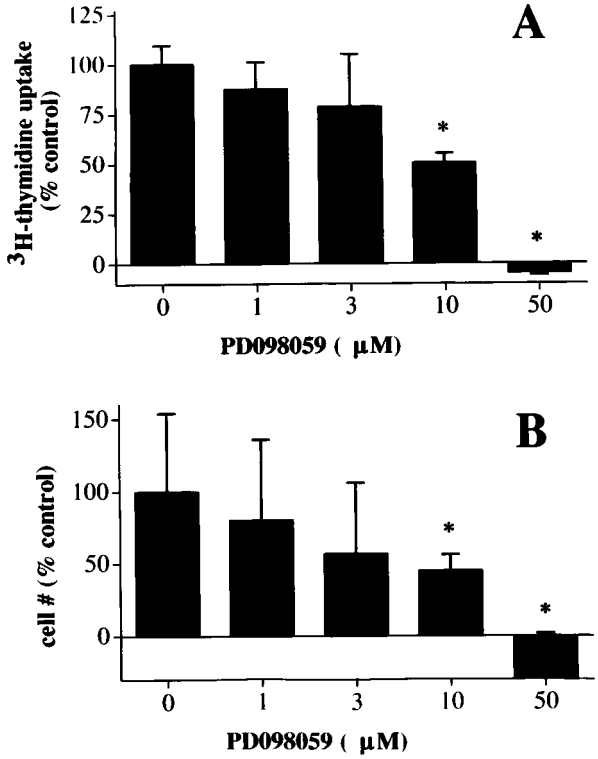

Fig. 3. Effect of PD098059 on SMC proliferation. Quiescent SMCs were stimulated with PDGF (5 ng/ml) in the presence of increasing concentrations of PD098059. SMC proliferation was determined by measuring **(A)** DNA synthesis by ^{3}H-thymidine incorporation, or **(B)** cell counts as described in Methods. Results are expressed as mean percent of control (PDGF-stimulated) proliferation ± SD. All experiments were repeated three times using cells derived from different saphenous veins. A representative example is displayed.

Chemotaxis requires early activation, and proliferation requires late activation of MAPK. Our data suggest that activation of MAPK leads to two distinct cellular events—migration and proliferation. In previous studies, we found that stimulation of SMCs with PDGF resulted in an early peak (10 minutes) followed by a persistent plateau in MAPK activity.[4] It has been postulated that a cascade of early cellular events results in cell migration. Conversely, studies in ours and other laboratories have shown that more delayed processes may be associated with cellular proliferation.[4,17] We, therefore, postulated that the initial peak of PDGF-induced MAPK activity is required for SMC chemotaxis and that the later plateau of MAPK activity is necessary for SMC proliferation.

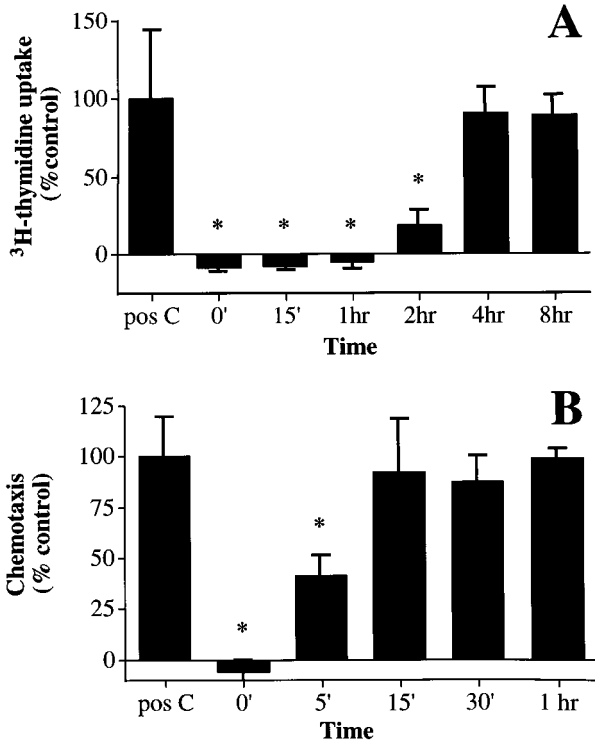

Fig. 4. Relationship between temporal activation of MAPK and SMC migration and proliferation. **A**, DNA synthesis was measured (as described in Methods) with PD098059 (50 μmol/L) added either simultaneously or at intervals after PDGF stimulation (5 ng/ml). Results are expressed as mean percent of control (PDGF-stimulated) proliferation ± SD. **B**, Microchemotaxis assays were performed (as described in Methods) with addition of PD098059 (50 μmol/L) simultaneously or at intervals after exposure of SMCs to PDGF (5 ng/ml). Results are expressed as mean percent of control (PDGF-stimulated) chemotaxis ± SD. All experiments were repeated three times using cells derived from different saphenous veins. Representative examples are displayed.

To test the first component of this hypothesis—that the early peak of MAPK activity is required for SMC chemotaxis—MAPK was inhibited by adding PD098059 (50 μmol/L) coincident with and at varying time points after stimulation of SMCs with PDGF-BB (Fig. 4, *B*). As previously demonstrated, simultaneous exposure of SMCs to PD098059 and PDGF completely blocked SMC chemotaxis. Chemotaxis was only partially inhibited if PD098059 was added 5 minutes after PDGF stimulation, suggesting that MAPK activity within the first 5 minutes is at least in part necessary to produce chemotaxis. If PD098059 was added at time points of 15 minutes or later after PDGF stimulation, chemotaxis was no longer inhibited. These findings suggest that MAPK activity that occurs greater than 15 minutes after PDGF stimulation is not required for SMC chemotaxis. Thus only MAPK activity that occurs within the first 15 minutes after PDGF stimulation is critical for the induction of SMC chemotaxis. Because MAPK activity peaks 10 minutes after stimulation of SMCs with PDGF, it appears that this early peak is necessary for the migratory effect of PDGF.

In similar experiments, we analyzed the relationship between the temporal course of MAPK activation and SMC proliferation. MAPK was inhibited using PD098059 (50 μmol/L) coincident with and at varying time points after PDGF stimulation of DNA synthesis (Fig. 4, *A*). SMC proliferation was completely eliminated when PD098059 was added at time points up to and including 1 hour after stimulation of SMCs with PDGF. Thus the MAPK activity within the first hour after PDGF stimulation was not sufficient to produce a proliferative response. If PD098059 was added at or greater than 4 hours after PDGF stimulation, proliferation was no longer inhibited. These findings suggest that MAPK activity that occurs greater than 4 hours after PDGF stimulation is not required for SMC proliferation. Thus only MAPK activity that occurs between 1 and 4 hours after PDGF stimulation appears to be required for the induction of SMC proliferation.

DISCUSSION

Both migration and proliferation of SMCs are prerequisite for the development of intimal hyperplasia, and both processes are simultaneously stimulated by growth factors such as PDGF. However, both processes are distinct, with migration requiring recruitment of integrins, focal adhesion proteins, and actin filaments, and proliferation requiring the activation of nuclear transcription factors that lead to the initiation of DNA synthesis. Because PDGF can stimulate both migration and proliferation, there must be divergence of the signaling pathway of this growth factor at some intracellular level beyond the receptor. The precise point of divergence has not been well elucidated. However, because MAPK activation can lead to both migration and proliferation, the division in the signaling pathways must be distal to MAPK and its upstream activators.

The selective inhibitor of MAPKK, PD098059, blocked both SMC migration and proliferation at concentrations similar to those required for the in vivo inhibition of MAPK in SMCs and in other cellular systems.[10] The finding that MAPK activation

mediates SMC proliferation is not surprising because this signaling protein is known to be associated with proliferation in numerous other cells. The role of MAPK activation in migration is less well understood.[7-9] Our studies using a specific inhibitor of MAPKK conclusively demonstrate that MAPK is *necessary* for migration after stimulation of SMCs with PDGF. However, these data do not establish whether activation of MAPK alone is *sufficient* to induce migration of human vascular SMCs. Interestingly, stimulation of SMCs with the potent growth factor bFGF produces a pattern of MAPK activation that is almost identical to that induced by PDGF.[4] If induction of MAPK activity was by itself sufficient to produce migration, it would therefore be anticipated that bFGF, like PDGF, would be a potent agonist of cell motility. However, we have previously found that bFGF stimulates little or no migration of human saphenous vein SMCs.[18] Therefore, a very strong early peak in MAPK activity, such as that induced by bFGF, does not necessarily result in SMC chemotaxis. It can be inferred from these findings that other signaling pathways must be activated in concert with MAPK for SMC migration to occur.

The finding that MAPK activation is necessary for both migration and proliferation raises the obvious dilemma of how one signaling protein can mediate two very distinct processes. Previous studies with MAPK in our laboratory revealed an association between prolonged MAPK activation after growth factor stimulation and the potential for the same growth factors to stimulate SMC proliferation.[4,5] For example, PDGF and bFGF are both potent agonists of SMC proliferation and both growth factors activate MAPK for periods up to 24 hours, whereas angiotensin II, which does not stimulate SMC proliferation, produces only an early peak in MAPK activity. Our current studies provide additional support for the hypothesis that the timing of MAPK activation determines its physiologic consequence. By adding the inhibitor PD098059 at intervals after stimulation of SMCs with PDGF, we were able to isolate the portion of MAPK activity that mediated both migration or proliferation. Inhibition of MAPK at any point during the first 15 minutes after PDGF stimulation either diminished or abolished SMC chemotaxis, whereas inhibition of MAPK beyond 15 minutes had no effect on SMC migration. From these findings, we can conclude that the early rise in MAPK activity that peaks at approximately 10 minutes is responsible for SMC chemotaxis. More prolonged activation of MAPK was required for SMC proliferation. Inhibition of MAPK at any point within the first hour after PDGF stimulation eliminated the SMC proliferative response, whereas the addition of the inhibitor after 4 hours had no effect. Thus MAPK activation between 1 and 4 hours is necessary for SMC proliferation.

In an interesting series of experiments, Duff and Berk[19] tested the hypothesis that prolonged activation of MAPK is associated with SMC proliferation. These authors found, as did we, that stimulation of SMCs with angiotensin II led to only a brief activation of MAPK and little SMC proliferation. By inactivating the MAPK phosphatase, MAPK phosphatase-1, these investigators artificially produced a situation in which stimulation of SMCs with angiotensin resulted in persistent MAPK activation. They predicted that cells treated in this manner would have an increased propensity to proliferate. However, the prolongation of MAPK activation that resulted from the elimination of MAPK phosphatase-1 led to diminished survival of SMCs; in fact, apoptosis was induced in these cells. These observations, although surprising, may still be compatible with our finding that MAPK activity between 1 and 4 hours is necessary for SMC proliferation. It is possible that a high level of MAPK activity sustained beyond 4 hours, as was the case with their model, is actually deleterious to SMCs.

The concept that temporal variations in MAPK activity might mediate distinct cellular events has been proposed by others. In the PC12 cell, the bimodal activation of MAPK is also associated with two distinct physiologic events. In contrast to our findings with SMCs, the early phase of MAPK activity appears to mediate proliferation of PC12 cells, and subsequent activity is associated with cell differentiation.[11] Susa et al.[17] have also proposed that cellular proliferation is dependent on late signaling events. For example, the second of two phases of protein kinase C activation, the phase stimulated by phosphatidylcholine, is responsible for cellular proliferation. Our data provide further support for the hypothesis that the timing of a signaling event is an important determinant of the subsequent cellular response, as we have demonstrated an association between early MAPK activation and SMC migration and later MAPK activation and cellular proliferation.

Multiple substrates for MAPK have been identified, many of which may potentially facilitate a proliferative cellular response. These include S6 kinase, the EGF receptor, and the transcription factors c-Myc, c-Jun, and c-Fos. The link between MAPK and the cellular mechanisms that underlie migration is

less clear. MAPK has been identified immunohistochemically in focal adhesion complexes after plating of fibroblasts onto various substrates implicating this protein in cytoskeletal activities such as attachment and migration.[20] Also, MAPK can phosphorylate and activate the calcium/calmodulin–dependent protein kinase, myosin light chain kinase. Klemke et al.[7] have shown that MAPK may effect cell motility in FG carcinoma and COS-7 cells by modulating this protein and its subsequent effect on myosin. Alternatively, Fox et al.[21] have postulated that bFGF may stimulate migration of endothelial cells through MAPK-mediated production of PLA_2 and arachidonic acid.

The upstream pathways that link growth factor receptors such as the PDGF receptor to MAPK have been better defined. Ligand attachment to the PDGF receptor leads to tyrosine phosphorylation of shc proteins inducing their association with the SH2 domain of the adapter protein Grb2. This protein complex then targets the nucleotide exchange factor SOS, which in turn associates with and activates the GTP-binding protein $p21^{ras}$. Ras activates the serine threonine kinase raf, which triggers a protein kinase cascade that leads to the activation of MAPK by the dual specificity protein kinase, MAPKK, which phosphorylates MAPK on threonine and tyrosine residues. The importance of upstream proteins such as raf and ras in cellular proliferation has been established. Although there appear to be multiple variations in the sequence of this upstream signaling cascade, this pathway appears to universally facilitate cellular proliferation in response to multiple growth factors. The necessity of this same signaling pathway for cell migration is less well established. Studies by Sosnowski et al.[22] and Fox et al.[21] have demonstrated a role for ras in the migration of endothelial cells. In these studies, injected oncogenic ras stimulated migration, and either a neutralizing ras-specific antibody or a ras-dominant interfering mutant protein inhibited migration. Kundra et al.[23] studied the importance of ras in the movement of SMCs and found that inhibition of ras blocked SMC migration, although surprisingly, overexpression of ras also inhibited migration. It is clear from these studies that $p21^{ras}$, which is upstream from MAPK, is involved in the signaling cascade that leads to migration, providing further support for our contention that the activation of the MAPK pathway is critical for SMC locomotion.

Recently, additional isotypes of MAPK have been identified, including a 38kD protein, p38/RK, and a 54 kd protein, SAPK/JNK.[15] These proteins are activated by inflammation, cytokines, and cellular stresses rather than growth factors, and they appear to have completely distinct, almost opposite, roles in cellular function. In most nonlymphoid cells, these two pathways function primarily to inhibit cell growth and to promote either necrotic or apoptotic cell death. It remains to be determined what role, if any, these new isotypes might play in SMC function, specifically proliferation and migration.

Accumulation of SMCs within the intima of the injured artery is essential for the development of intimal hyperplasia, and both SMC migration and proliferation contribute to this process. The relative importance of these two events is not clear; however, inhibition of both migration and proliferation would provide a potent means for preventing the development of hyperplastic lesions. We have learned that MAPK is an essential intermediate in the intracellular signaling pathways that mediate both SMC migration and proliferation. Moreover, different phases of MAPK activation are responsible for these two distinct events. Although our findings thus far are from in vitro studies, we believe that the MAPK inhibitor, PD098059, could potentially be used to selectively inhibit intimal hyperplasia and control this process that so greatly limits the longevity of vascular reconstructions.

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